Targeted Quantum Dot Conjugates for siRNA Delivery

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Treatment of human diseases such as cancer generally involves the sequential use of diagnostic tools and therapeutic modalities. Multifunctional platforms combining therapeutic and diagnostic imaging functions in a single vehicle promise to change this paradigm. in particular, nanoparticle-based multifunctional platforms offer the potential to improve the pharmacokinetics of drug formulations, while providing attachment sites for diagnostic imaging and disease targeting features. We have applied these principles to the delivery of small interfering RNA (siRNA) therapeutics, where systemic delivery is hampered by rapid excretion and nontargeted tissue distribution. Using a PEGlyated quantum dot (QD) core as a scaffold, siRNA and tumor-homing peptides (F3) were conjugated to functional groups on the particle's surface. We found that the homing peptide was required for targeted internalization by tumor cells, and that siRNA cargo could be coattached without affecting the function of the peptide. Using an EGFP model system, the role of conjugation chemistry was investigated, with siRNA attached to the particle by disulfide cross-linkers showing greater silencing efficiency than when attached by a nonreducible thioether linkage. Since each particle contains a limited number of attachment sites, we further explored the tradeoff between number of F3 peptides and the number of siRNA per particle, leading to an optimized formulation. Delivery of these F3/siRNA−QDs to EGFP-transfected HeLa cells and release from their endosomal entrapment led to significant knockdown of EGFP signal. By designing the siRNA sequence against a therapeutic target (e.g., oncogene) instead of EGFP, this technology may be ultimately adapted to simultaneously treat and image metastatic cancer.

INTRODUCTION

The development of multifunctional nanoparticles for treatment of focal disease is attractive for several reasons: they exhibit unique pharmacokinetics including minimal renal filtration, they have high surface to volume ratios enabling modification with surface functional groups that can be used to specifically target the delivery of therapeutic agents to sites of disease, and they can serve as vehicles for integration of diagnostic imaging and therapeutic drug delivery, a potentially transformative clinical paradigm. Use of a nanoparticle imaging core that is decorated with functional moieties provides a strategy that is particularly amenable to modular design of a multifunctional nanoparticle where features may be interchanged or combined to tailor formulations for a plethora of applications. In an attempt to move toward this goal, we have previously combined peptides derived from phage display- a powerful biological screening technique- with fluorescent semiconductor quantum dots to target multivalent nanoparticles to tumors (*1*). In this report, we further explore the feasibility of incorporating an oligonucleotide-based therapeutic cargo, siRNA. Short, double-stranded small-interfering-RNAs (siRNA) are one manifestation of a phenomenon known as RNA interference whereby translation of a target protein is inhibited. This type of therapeutic cargo is of particular interest in recent years because it has the potential to modulate so-called 'nondruggable' targets (*2*, *3*).

The *untargeted*, systemic delivery of siRNA has been explored by conglomeration of duplexes into nanosized complexes that reduce their renal filtration rates, extending the circulation half-life well beyond the ∼6 min observed for unmodified siRNA (*4*). For example, cholesterol−siRNA conjugates bind serum albumin after intravenous injection, forming long-circulating "natural" nanoparticles (*4*). Similarly, siRNA−carrier complexes can be formed ex vivo, prior to injection, by condensing the nucleic acid with a cationic protein (e.g., protamine (*5*)) or polymer (e.g., poly(ethylene imine) (PEI) (*6*), cyclodextrin-containing polycations (*7*), or PEG-based block cationmer (*8*)). In addition, targeted delivery of such agents has the potential to limit collateral toxicity and 'off-target' effects. Targeting has been explored through use of through the attachment of antibodies (*5*), small molecules (e.g., transferrin (*7*)), aptamers (*9*, *10*), or well-established peptide ligands (*6*); however, these approaches are typically not modular nor multifunctional (i.e., do not incorporate imaging moieties). Addition of a nanoparticle-based imaging agent to siRNA delivery strategies may be particularly advantageous as protein knockdown by RNAi is delayed (>48 h or more after administration), and many fluorescent dyes are not stable for monitoring delivery over extended periods of time in vivo. In vitro, co-delivery of fluorescent reporter plasmid along with the siRNA is often utilized; however, this is unlikely to be used clinically given the potential risks associated with integration into host DNA.

Quantum dots offer the potential to serve as photostable beacons to track siRNA delivery. We have previously explored their utility for monitoring siRNA delivery in vitro by co-complexing QDs with cationic liposomes and siRNA (*11*, *12*); however, this approach is not amenable to either systemic delivery, because of their relatively large size and rapid uptake

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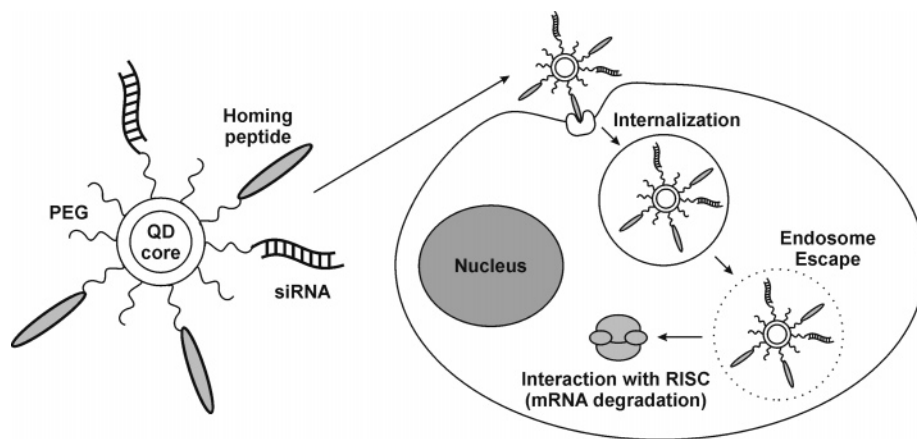
Figure 1. Design of a multifunctional nanoparticle for siRNA delivery. Because of their photostable fluorescence and multivalency, QDs are suitable vehicles for ferrying siRNA into live cells in vitro and in vivo. Conjugation of homing peptides (along with the siRNA cargo) to the QD surface allows targeted internalization in tumor cells. Once internalized, these particles must escape the endolysosomal pathway and reach the cytoplasm to interact with the RNA-induced silencing complex (RISC), which leads to degradation of mRNA homologous to the siRNA sequence.

by macrophages, or targeted delivery, as they are ubiquitously internalized. In this report, we sought to generate multifunctional conjugates of a size compatible with eventual systemic delivery ($5 < d < 200$ nm) upon a quantum dot core that presented both targeting peptides and siRNA (Figure 1). A QD core with emission in the near-infrared (NIR) was chosen to allow greater imaging depth in tissue than that achieved with visible wavelengths. The siRNA was attached to the surface with a chemical cross-linker, rather than the electrostatic interactions used in many schemes. This strategy grants a smaller overall size than polymer-based particles, which may carry advantages in evading uptake by the reticuloendothelial system (*13*). As a proof-of-concept, the attached siRNA was designed against the enhanced green fluorescent protein (EGFP) gene, and complexes were delivered to EGFP-transfected HeLa cells in vitro. A tumor-homing peptide (F3), targeting cell-surface nucleolin (*14*), was attached to achieve cell uptake. F3 peptide has been shown to target tumor cells in vitro and in vivo when administered as a free peptide and also when coconjugated with poly(ethylene glycol) to quantum dots delivered systemically in mice bearing xenograft tumors(*15*).

Combining these components onto a single particle brings several engineering requirements and constraints that affect their design and fabrication. Accordingly, in this report we first investigate whether the attachment of the peptide is necessary and sufficient to achieve cell internalization. As particle surface area is limited (~100 surface amines on these QDs), the actual copy numbers of targeting peptide and siRNA required are also quantified. Increasing the number of peptides on the QDs lowers the siRNA dose per particle and vice versa. Next, because conjugation of siRNA to the particles must be stable in cell media yet also allow the duplex to interact with the RNA-induced silencing complex (RISC) in the cytosol, we test the efficacy of two different cross-linkers for this task. While RISC binding may be possible with the duplex attached to the particle, release of the siRNA from the particle's surface upon internalization proved advantageous to knockdown efficacy. Finally, cytoplasmic delivery, free of endosomes, is essential to allow siRNA to interact with cellular silencing machinery. While fluorophore-labeled F3 peptide has been shown to reach the nuclei of tumor cells (*15*), internalization of particles generally follows the endolysosomal pathway. In this report, the addition of endosome-disrupting agents was required to aid escape and increase siRNA-mediated knockdown.

EXPERIMENTAL SECTION

Materials. Quantum dots with emission maxima of 655 or 705 nm and modified with PEG and amino groups were obtained from Quantum Dot Corporation (ITK amino). QD concentrations were measured by optical absorbance at 595 nm, using extinction coefficients provided by the supplier. Cross-linkers used were sulfo-LC-SPDP (sulfosuccinimidyl 6-(3′-[2-pyridyldithio]-propionamido)hexanoate) (Pierce) and sulfo-SMCC (sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate) (Sigma). Synthetic RNA duplexes directed against the EGFP mRNA were synthesized, with the sense strand modified to contain a 5′ thiol group (Dharmacon) (sense: 5′-Th-$(CH_2)_6$-GGC UAC GUC CAG GAG CGC ACC; antisense: 5′-UGC GCU CCU GGA CGU AGC CUU). The F3 peptide was synthesized with an aminohexanoic acid (Ahx) spacer and cysteine residue added for conjugation (final sequence: C[Ahx]-AKVK DEPQR RSARL SAKPA PPKPE PKPKK APAKK). A FITC-labeled F3 peptide was also synthesized, along with KAREC (Lys-Ala-Arg-Glu-Cys), a five amino acid control peptide. All peptides were synthesized by *N*-(9-fluorenyl-methoxycarbonyl)-L-amino acid chemistry with a solid-phase synthesizer and purified by HPLC. The composition of the peptides was confirmed by MS.

Conjugation of Peptides and Nucleic Acid to QDs. Amino-modified QDs were conjugated to thiol-containing siRNA and peptides using sulfo-LC-SPDP and sulfo-SMCC cross-linkers. QDs were resuspended in 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.2, using Amicon Ultra-4 (100 kDa cutoff) filters. Cross-linker (1000-fold excess) was added to QDs and allowed to react for 1 h. Samples were filtered on a NAP-5 gravity column (to remove excess cross-linker) into similar buffer supplemented with 10 mM EDTA. siRNA was treated with 0.1 M DTT for 1 h and filtered on a NAP-5 column into EDTA-containing buffer. Peptides were typically used from lyophilized powder. Peptide and/or siRNA was added to filtered QDs and allowed to react overnight at 4 °C. Using three Amicon filters, product was filtered twice with Dulbecco's phosphate-buffered saline (PBS), twice with a high salt buffer (1.0 M sodium chloride, 100 mM sodium citrate, pH 7.2), and twice again with PBS. High salt washes were required to remove electrostatically bound siRNA and peptide, which was not removed with PBS washes alone.

For siRNA−QDs, a 10-fold excess of siRNA was typically used for both cross-linkers. In the case of sulfo-LC-SPDP, the amount of conjugated siRNA was assayed using gel electro-

phoresis (20% TBE gel, Invitrogen), staining with SYBR Gold (Invitrogen). To confirm that similar amounts of siRNA (approximately two per QD) were conjugated to QDs using sulfo-SMCC, particles were stained with SYBR Gold and measured with a fluorimeter (SpectraMax Gemini XS, Molecular Devices).

For F3/siRNA−QDs and KAREC/siRNA−QDs, a molar ratio of 15:70:1 (siRNA:peptide:QDs) was found to be optimum, though a variety of ratios were attempted (Figure 4A). These conditions yielded approximately 20 F3 peptides and 1 siRNA duplex per particle.

Cell Culture. Internalization and knockdown experiments were performed using a HeLa cell line transfected with destabilized EGFP with a half-life of 1 h (courtesy of Phillip Sharp, MIT). Growth media was Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and supplemented with 10% FBS, 100 units/mL penicillin, 100 ug/mL strepto-mycin, and 292 ug/mL L-glutamine. Cells were passaged into 24-well plates and used at 50−80% confluency for internaliza-tion experiments and 20−40% confluency for knockdown experiments.

For internalization experiments (Figure 2), QDs were added to cell monolayers in media without serum at a final concentra-tion of 50 nM. After 4 h, cells were washed with media, treated with trypsin (0.25%) and EDTA, and resuspended in 1% BSA (in PBS) for flow cytometry (BD FACSort, FL1 for EGFP signal and FL3 for QD signal). Fluorescence data on 10 000 cells was collected for each sample, and the geometric mean of intensity was reported.

For knockdown experiments in Figure 3, siRNA−QDs (in 50 uL serum/antibiotic-free media) were added to Lipofectamine 2000 (1 μL in 50 μL media, Invitrogen) and allowed to complex for 20 min. Cell media was changed to 400 μL of serum/ antibiotic-free per well, and QD solutions (100 μL) were added dropwise. Complete media was added 12−18 h later, and 48 h after the QD were added, cells were trypsinized and assayed for fluorescence by flow cytometry.

To assess EGFP knockdown, 50 nM or 10 nM concentrations of F3/siRNA−QDs or KAREC/siRNA−QDs were added to cell monolayers (20−40% confluent) in media with serum/antibiot-ics. To demonstrate specificity, irrelevant siRNA (designed against the Lamin A/C gene, as described in ref 12) was conjugated to QDs along with F3 and also delivered to cells. Four hours later, cells were washed with similar media. Some samples were then treated with 1 μL of Lipofectamine per well (added dropwise in 100 μL media) either immediately after washing or after a 90 min incubation at 37 °C (to allow membrane recycling). For all samples, media was changed to complete DMEM with serum/antibiotics ∼16 h after the addition of QDs and assayed by flow cytometry 48 h from the start of the experiment. For imaging, cells were initially seeded on glass-bottom dishes (Mat-Tek) and observed 48 h after the addition of QDs using a 60× oil immersion objective. Images were captured with a SPOT camera mounted on a Nikon TE200 inverted epifluorescence microscope.

RESULTS AND DISCUSSION

Taking a modular approach, particle internalization and siRNA attachment were investigated separately before these functions were combined in a single particle. First, peptides were conjugated to QDs to improve tumor cell uptake. Addition of as-purchased PEGlyated QDs to HeLa cell monolayers led to minimal cell uptake, as quantified with flow cytometry (Figure 2A). Conjugation of siRNA or a control pentapeptide (KAREC) did not increase QD internalization, but addition of F3 peptide to the QDs improved the uptake significantly (2 orders of magnitude). To confirm the specificity of F3 uptake, free F3
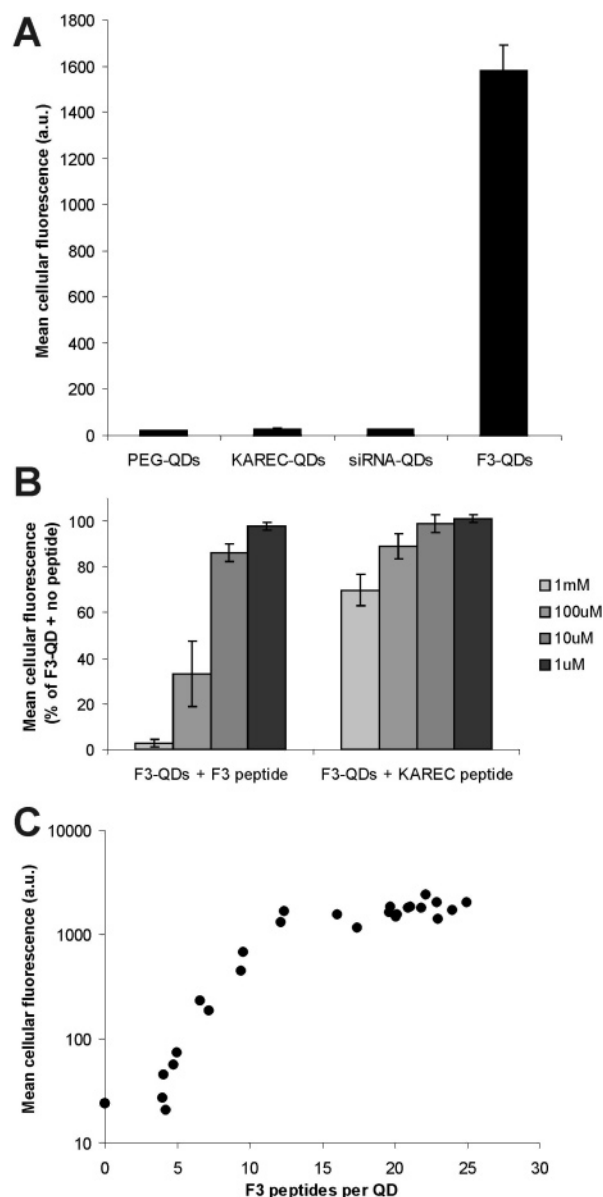

Figure 2. Attachment of F3 peptide leads to QD internalization in HeLa cells. Thiolated peptides (F3 and KAREC control) and siRNA were conjugated to PEG-amino QD705 particles using sulfo-SMCC. Particles were filtered to remove excess peptide or siRNA and incubated with HeLa cell monolayers for 4 h. Flow cytometry indicated the F3 peptide is required for cell entry (A). The addition of free F3 peptide inhibits F3−QD uptake, while KAREC peptide does not, suggesting the F3 peptide and F3-labeled particles target the same receptor (B). In part C, the relationship between the number of F3 peptides per QD and cell uptake was examined. In these experiments, FITC-labeled peptide was conjugated to QDs using sulfo-LC-SPDP. For each formulation (black circles), peptide:QD ratio was determined by measuring the QD concentration by absorbance and then treating the conjugate with 2-mercaptoethanol, filtering out the QDs, and measuring the FITC fluorescence. Cell uptake increases dramatically with peptide number but appears to saturate around 10−15 F3s per QD.

peptide was added to cells along with 50 nM F3−QDs (Figure 2B). Dose-dependent inhibition of uptake was observed with F3 peptide concentrations from 1 μM to 1 mM. Inhibition of uptake by an irrelevant peptide, free KAREC, was minimal by comparison. The large excess of free peptide required for inhibition may be due to multiple copies of the F3 peptide on each QD and improved receptor binding as a result of multi-valency.

To quantify the number of peptides added per particle, FITC-labeled F3 peptide was synthesized and attached to QDs using
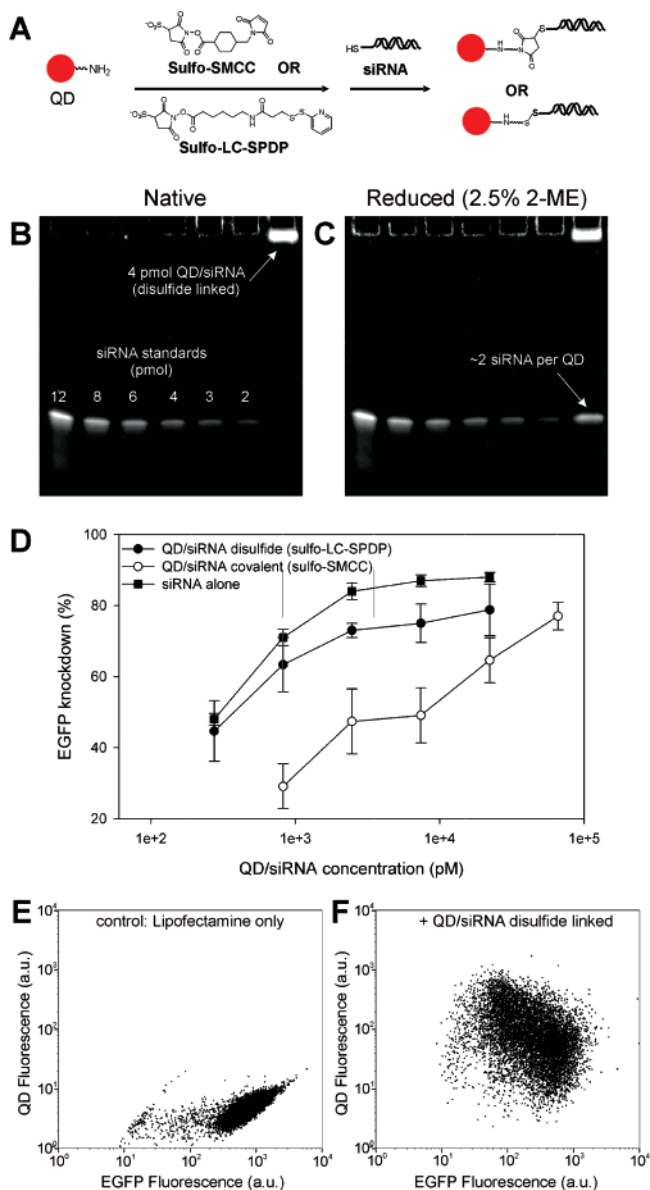
Figure 3. Conjugation of siRNA to QDs with cleavable or noncleavable cross-linkers. Thiol-modified siRNA was attached to PEG-amino QDs using the water-soluble heterobifunctional cross-linkers sulfo-SMCC and sulfo-LC-SPDP (A). The cross-link produced by SPDP is cleavable with 2-mercaptoethanol (2-ME), while SMCC yields a nonreducible linkage. Gel electrophoresis of the disulfide-linked conjugates indicated that no siRNA are electrostatically bound to the conjugate (B). Upon treatment with 2-ME, the QD/siRNA cross-link is reduced and the siRNA migrated down the gel alongside siRNA standards (C). QD/siRNA conjugates (or siRNA alone) were delivered to EGFP-expressing HeLa cells using Lipofectamine 2000 (cationic liposome reagent). Cells were trypsinized and assayed by flow cytometry 48 h later. Comparison with control cells (treated with Lipofectamine alone) indicated the disulfide bond leads to superior EGFP knockdown (% reduction in geometric mean fluorescence) (D). Comparing a dot-plot of cells treated with Lipofectamine alone (E) or disulfide-linked QD/siRNA (F) revealed a negative correlation between QD uptake and EGFP signal. Thus, the QD label can serve as a means of quantifying siRNA delivery and thus knockdown.

a cleavable cross-linker (sulfo-LC-SPDP). After unreacted peptide was removed by filtration, 2-mercaptoethanol (2-ME) was added to reduce the disulfide bond between peptide and QD. Using a 100 kDa cutoff filter, F3-FITC peptide was separated from the QDs and quantified by fluorescence. Several reactions were performed with various amounts of FITC-F3 and siRNA as reactants. For each formulation, the cellular uptake was quantified by flow cytometry and F3 number measured

(Figure 2C, each point indicates a separate formulation). Attachment of a small number of peptides (0−5) did not lead to significant uptake (less than 10% of maximum). Uptake increases with peptide number but begins to saturate around 15 copies per QD.

The use of cleavable (sulfo-LC-SPDP) or noncleavable (sulfo-SMCC) cross-linkers for the attachment of F3 peptide did not significantly affect cell uptake. The choice of cross-linker, however, may affect the ability of the siRNA cargo to interact with RISC. The interior of the cell is a reducing environment, which would lead to cleavage of the disulfide bond generated by sulfo-LC-SPDP, freeing the siRNA. On the other hand, the amide bond produced by sulfo-SMCC is unaffected by reducing conditions (confirmed by treating the conjugates with 2.5% 2-ME for 30 min), leaving the intracellular QD/siRNA conjugate intact. We compared the efficiency of QD/siRNA conjugates prepared with both cross-linkers using an EGFP model system. Delivery of the conjugates to EGFP-labeled HeLa cells was performed by first complexing the particles with a cationic liposome transfection reagent (Lipofectamine 2000), to satisfy the functions of cell internalization and endosome escape, and knockdown efficiency was quantified by a reduction in EGFP fluorescence over controls (Lipofectamine only).

Using gel electrophoresis, the amount of siRNA conjugated per particle was quantified relative to double-stranded RNA standards. Particles conjugated using sulfo-LC-SPDP were first introduced under native (nonreduced) conditions (Figure 3B). The absence of a siRNA band in the QD/siRNA lanes indicates that no siRNA is noncovalently bound to the particles. Exposing the particles to 2-ME for 30 min led to the appearance of a siRNA band in the SPDP lane, which was quantified with RNA standards and ImageQuant software. Using this approach, approximately two siRNA duplexes were conjugated per QD under these conditions (Figure 3C). Cellular fluorescence was quantified 48 h after incubation with HeLa cells using flow cytometry. As hypothesized, the QD/siRNA formulation produced with the disulfide bond (using sulfo-LC-SPDP) led to greater EGFP knockdown (Figure 3D). The level of knockdown attained with disulfide-linked QD/siRNA, however, was less than observed when an equal concentration of free siRNA was delivered with Lipofectamine. On the basis of previous observations(*12*), one potential cause may be the limited surface area of the cationic liposomes, which is shared by QDs and siRNA. Additionally, the presence of the QDs in the endosome may reduce the efficiency of escape, or reduction of the disulfide bond may be incomplete, resulting in less efficient complexation with RISC.

The F3:siRNA reaction ratio was varied with the goal of generating a formulation capable of high cell uptake as well as the ability to carry a significant payload of siRNA. The cleavable cross-linker allowed the removal and quantification of both species after F3 peptide and siRNA coattachment. The results indicate a tradeoff between one siRNA per particle with high uptake (>15 peptides) and two duplexes but lower uptake (<10 peptides) (Figure 4A). Negatively charged siRNA may be electrostatically adsorbing to the surface of the aminated QDs, preventing the attachment of additional F3 peptides. Potentially, performing the reaction in high salt conditions, or in the presence of a surfactant, may allow higher loading. Since both high uptake efficiency and siRNA number are required for knockdown, particles with ∼20 F3s and a single siRNA duplex were further investigated in cell studies.

When incubated with cells, these 20-F3/1−siRNA−QDs were shown to internalize significantly but did not lead to reduction in EGFP fluorescence 48 h later. Fluorescence microscopy revealed that the particles were intracellular, but they colocalized with an endosomal marker (LysoSensor, Molecular Probes).
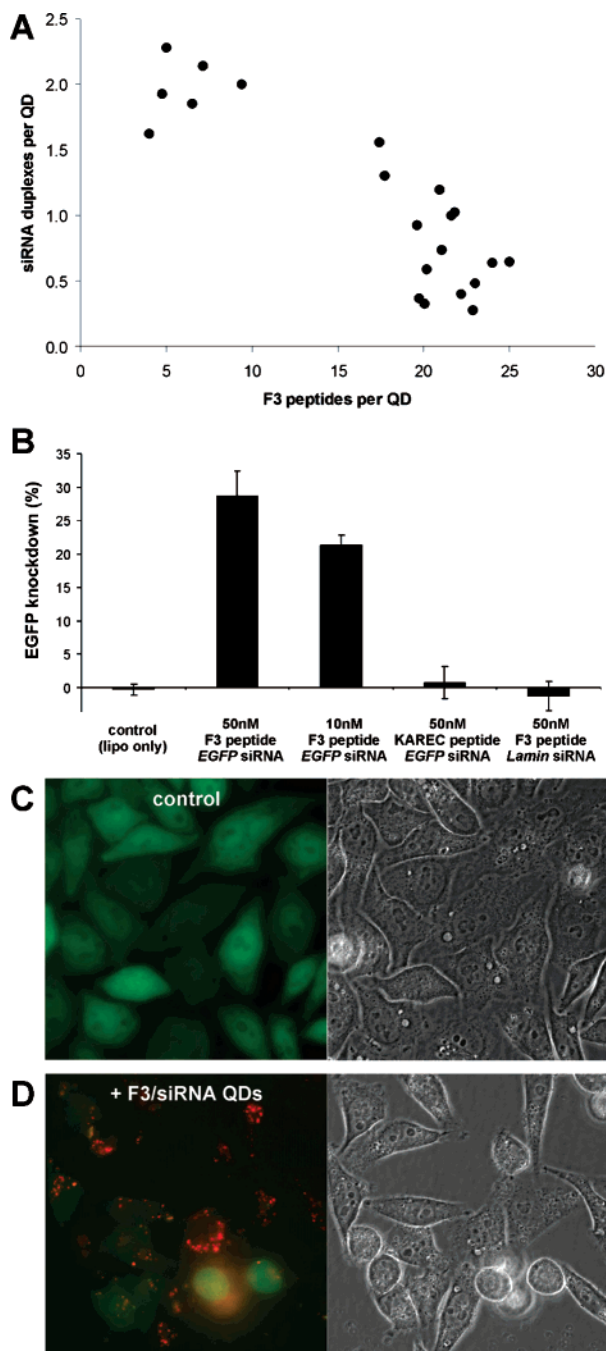
Figure 4. Co-attachment of F3 peptide and siRNA cargo allows EGFP knockdown upon delivery and endosome escape. Because of a limited number of attachment sites on the QDs, the goal of coattachment was to maximize siRNA loading while conjugating sufficient F3 peptides to allow internalization (>15). Varying the F3:siRNA ratio resulted in a number of QD formulations (black circles, A). We chose a formulation containing ~20 F3 peptides and ~1 siRNA per QD for knockdown studies in cell monolayers (B−D). EGFP-expressing HeLa cells were treated with 50 nM F3/siRNA−QDs for 4 h and then washed with cell media. When assayed for green fluorescence 48 h later, no knockdown was observed ("control", B). When these cells were treated with cationic liposomes (Lipofectamine 2000) immediately after removing the QDs and washing, a ~29% reduction in EGFP was observed. A lower concentration of QDs (10 nM) is less effective (21% knockdown). Incubation with KAREC-labeled particles followed by cationic liposomes leads to minimal particle internalization and thus no knockdown. Attachment of an irrelevant siRNA (directed against Lamin A/C) to QDs along with F3 peptide also results in no knockdown when delivered to cells followed by lipofectamine. Fluorescence imaging of cells incubated with F3/siRNA QDs (red dots) showed a reduced green fluorescence (D), compared with control cells incubated with Lipofectamine alone (C).

Addition of an endosome escape agent, therefore, was required to achieve knockdown. Specifically, after incubation of cells with F3/siRNA−QDs and washing, cationic liposomes were added for 12 h. Although cationic liposomes and polymers are typically used to form complexes with nucleic acids or particles, thereby ferrying the payload inside cells, in this case the reagent leads to endosomal escape of previously internalized QDs. We hypothesize that the cationic liposomes are internalized into new endosomes, which fuse with the endosomes carrying the QDs. As the pH of the vesicle is lowered by the cell, osmotic lysis leads to the release of both species into the cytoplasm. To assess the importance of the targeting ligand, particles carrying siRNA and a control peptide (KAREC) were used. These KAREC/siRNA particles were not internalized, and no EGFP knockdown was observed, despite endosome disruption. The specificity of knockdown was verified by delivery of F3/siRNA QDs containing an irrelevant siRNA (directed against Lamin A/C). Additionally, a time lag of 90 min between washing the cells free of QDs and cationic liposome addition did not lead to significant reduction in efficiency, indicating that endosomal degradation of the siRNA is not an issue on this time scale.

In addition to cationic liposomes, some chemotherapeutics, such as chloroquine have been shown to be capable of endosomal escape (*16*). While an endosome escape step could be a realistic part of a treatment regimen, there is also potential that this function could be built into each particle. Addition of fusogenic moieties to the QD surface, for example, may further improve delivery of the multifunctional particles described (*17*).

CONCLUSIONS

Decorating the surface of a fluorescent quantum dot with both a targeting ligand and siRNA duplex requires a tradeoff in the number of each species but can be used to generate a conjugate capable of knockdown in vitro. We found that multiple copies of the F3 targeting peptide were required for QD uptake, but that siRNA cargo could be co-attached without affecting the function of the peptide. Disulfide (sulfo-LC-SPDP) and covalent (sulfo-SMCC) cross-linkers were investigated for the attachment of siRNA to the particle, with the disulfide bond showing greater silencing efficiency. Finally, after delivery to cells and release from their endosomal entrapment, F3/siRNA−QDs produced significant knockdown of EGFP signal. By designing the siRNA sequence against a therapeutic target (e.g., oncogene) instead of EGFP, the technology explored in this study may be adapted to treat and image diseases such as cancer. This technology could also be readily adapted to other nanoparticle platforms, such as iron oxide or gold cores, which allow image contrast on magnetic resonance or X-ray imaging, respectively, and may therefore mitigate concerns over QD cytotoxicity and the limited tissue penetration of light. QDs, however, remain an attractive tool for in vitro and animal testing, where fluorescence is the most accessible and common imaging modality.

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